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DISEASE PROCESSES IN SEASTARS: A METCHNIKOVIAN CHALLENGE*

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Abstract

The adult seastar is an ideal model for study of phagocytosis and inflammation. The behavior of the circulating amebocytes responsible for phagocytosis and clumping can be directly observed in the coelomic fluid circulating in the transparent papulae of the limbs. Intracoelomic injection of an autologous amebocyte extract induces temporary, reversible, clumping and adherence of amebocytes. Injected, foreign, red-pigmented sea urchin amebocytes are phagocytosed by seastar amebocytes which then form clumps in the tips of the papulae and traverse to the external surface through penetrating lesions. Successive injections may cause acute inflammation and edema. During the acute inflammatory response, a substance(s) is released into the coelomic fluid which stimulates the urn cell complex of *Sipunculus nudus* to secrete mucus *in vitro*; the substance disappears from the fluid by 24 hours. A spontaneous ciliate infection of seastar testes causes failure of the infected animal's amebocytes to clump on glass or plastic. Seastars recognize foreign grafts, but the role of amebocytes in the rejection has not been shown to be explicit. In the separate water vascular system of seastars, a secondary inflammatory response (amebocyte clumping) is induced by injections of bacterial suspensions and by repeated injection of sea urchin amebocytes into the coelom. Heavy exposure of seastars to ciliate populations induces appearance of a lysin which is not derived from circulating cells.

Introduction

Although Metchnikov** (1892) first tested his basic idea concerning phagocytosis and inflammation in seastar larvae, the mature seastar has been used only sporadically as an experimental animal for the study of inflammation. It is the purpose of this informal review to describe some recent experiments which show that inflammatory changes may be easily followed in the transparent papulae of the adult seastar. Unique changes in macrophage (amebocyte) behavior are also induced by spontaneous disease in the adult animal.

In the years following Metchnikoff's observations of spontaneous phagocytosis of diatoms and debris by *Bipinnaria larvae* (Metschnikoff, 1883) and the famous

Abbreviation: MSS, mucus-stimulating substance.

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** For the spelling of the name Metchnikoff, each of the original references was used.

Editor's Note: Following solicitation of an article in the area of invertebrate pathobiology, Dr. Bang submitted a draft outline of this paper for the Editor's consideration. After Dr. Bang's untimely death on October 3, 1981, Mrs. Betsy Bang prepared the formal manuscript for publication.

experiment with the rosethorn (Metchnikoff, 1905), there were apparently very few further pathological studies on seastar larvae. Metalnikov and Rapkine (1925) reported in the *Comptes Rendus* that injection of India ink into 16–18 hour blastulas of sea urchins (*Paracentrotus lividus*) was followed by phagocytosis of the ink 3–4 hours later. Similar results were obtained with three species of bacteria, although *Vibrio cholerae* killed these larvae. Since that time, embryologists have frequently noted the ingestion of foreign material by larvae, but as far as I know there has been no systematic study of these processes. The transparent two-day old *Asterias* larva, which is capable of ingesting small clams (Mead, 1899) might be an ideal subject.

In contrast to what has been done on the structure and physiology of echinoderms (Cuénot, 1948; Hyman, 1955; Boolootian, 1966), there is only a scattering of information on pathological changes in the adult seastar. Within this context, we will describe the three circulatory systems of *Asterias* which have been used for experimental studies, and then describe different phenomena which directly affect the circulating amebocytes found in the coelomic cavity and in the water vascular system. In Metchnikovian terms, the transparency of intact animals is of great advantage for continuous observation of changes in individual experimental animals, and spontaneous infections offer visible insights into experiments of nature.

Results and Discussion

The seastar as experimental animal

The phenomena which we have examined in adult seastars are: (i) reversible clumping of the amebocytes in the papulae; (ii) normal clumping of the amebocytes when they are placed on glass, and inhibition of this clumping in amebocytes taken from seastars which have a natural ciliate infection in the testes (less frequently in the ovaries); (iii) recognition of foreignness of experimentally injected material followed by phagocytosis and emigration of phagocytosed material through the papulae; and (iv) rejection of foreign grafts, either of the hepatopancreas or of the skin. In all cases, the amebocyte is probably involved, but lytic factors have also been demonstrated under certain conditions.

Two important precautions are essential in working with adult seastars experimentally. First, animals must be healthy. Stars that have been dragged across the sea bottom on a mop during dredging lose a great deal of surface skin and usually have marine bacteria circulating within the coelom; many will subsequently autotomize one or more limbs (Bang and Lemma, 1962). Therefore, the animals must be collected by hand. In the Woods Hole area, *Asterias forbesi* can be handcollected by divers. In northern France, *Asterias rubens* can be collected directly off rocky mussel beds at low tide. The coelomic fluid of healthy animals is free of bacteria, and clumping is absent from the papulae.

Secondly, transparency varies with the species and age of an animal. *Asterias forbesi* which weigh 50 g or more do not have transparent feet, but in 6–10 g stars circulating cells are readily seen as they are driven by the internal cilia in the papulae and the feet. Present-day dissecting microscopes, with intense, relatively cold, lights, reveal to the practiced observer a wealth of circulating cells rushing through these respiratory and excretory papulae and feet.

I. Anatomy

For the present purpose, a review of only the simplest aspects is necessary. A cross-section of a limb shows the papulae emerging all over the surface, predom-

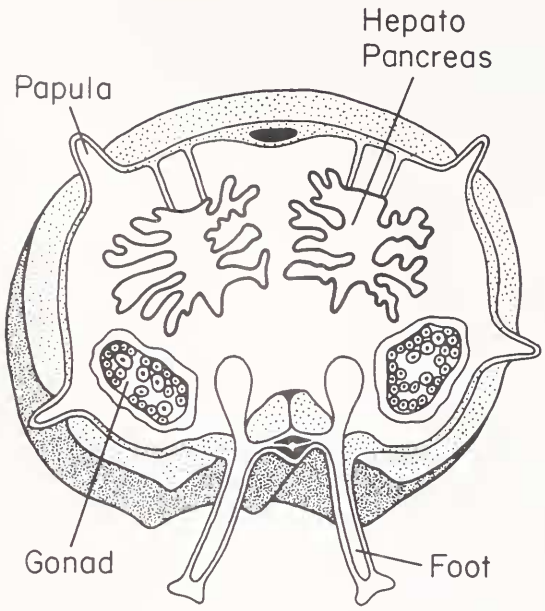

FIGURE 1. Cross-section of a seastar.

inantly on the dorsal or aboral surface (Fig. 1). These have a layer of epidermal cells and endodermal cells, both of which are ciliated, and a mesenchymal layer which contains contractile muscle cells. The papulae may be contracted or relaxed either in response to their own muscular action or to the general pressure of the body wall. We have often seen a general collapse of the usually distended papulae of a 10 g seastar immediately after a second injection of a foreign substance such as ciliate-infected crab blood, or sea urchin blood. In extreme cases, the change in pressure pulls the inverted papulae into the coelomic cavity. The papulae may also be grossly distended during edema. The coelomic fluid of normal *Asterias* contains only one type of cell, the petaloid cell, which circulates everywhere in response to both muscular movements of the whole arm and the constant directional beating of the cilia. The water vascular system is separate. Injection of particulates, such as India ink or carmine powder, into the coelom is not followed by their appearance in this second system. Even dyes, such as Evans' blue (Noble and Gregerson, 1946), penetrate from the coelom into the feet only after a number of days, and some of this may be by cell transfer. The third system, the axial organ and the hemal canals, is less well understood and reference to their role in pathology will be incidental.

II. Responses to injury

The use of natural responses to injury or infection among invertebrates was a hallmark of Metchnikoff's work on invertebrates. Not only was the yeast infection of *Daphnia* (Metschnikoff, 1884) studied extensively, but even during the original study of the metamorphosis and development of seastar larvae, he was careful to pay attention to the presence of foreign material that had been ingested by the larvae during their residence in the sea (Metschnikoff, 1883).

TABLE I

Prevalence of non-clumping amebocytes among Asterias *during summer 1976.*

Month/date	Number tested	Number negative for clumping	Per cent negative
6/21–23	38	2	5.3
6/29–30	40	3	7.5
7/ 6–12	50	18	36.0
7/22–8/4	126	6	5.0
8/11–14	179	3	1.7
8/17–19	81	8	10.0
8/19–23	197	0	0.0
9/ 3–6	110	1	0.9

a. Clumping. Some years ago, the fact that traumatized stars often showed clumps of cells circulating in the papulae and that in cases of severe trauma these clumps may be tightly contracted and become attached closely to the inside of the papulae, led us to make crude extracts of the amebocytes and to inject the fluid extract into the coelomic cavity (Bang and Lemma, 1962; Bang, 1975). Direct observation of the papulae showed an initial loose aggregation of the cells, later clumping and contraction of these aggregates, and the final tight adherence of balls of amebocytes to the inside of the papulae. This phenomenon occurred within 2–5 minutes, lasted only 15–20 minutes, and then disappeared, leaving the animals susceptible to repeated stimuli of the same kind without any apparent change in sensitivity.

b. Failure of amebocytes to respond to glass. A well described ciliate infection of the gonads (especially of the testes) of seastars was first observed in *Asterias rubens* by Cépède (1910), subsequently studied in *Asterias forbesi* in the U.S. by Smith (1936), and in England by Vevers (1951). It attains sufficient frequency under certain conditions that it was seriously considered by Galtsoff and Loosanoff (1939) as a method of controlling seastars which prey on shellfish beds. The apparently indirect effect of the infection on the glass-induced clumping of seastar amebocytes was discovered by one of my students in 1970, when he found that the routine clumping of cells on glass did not occur in animals in which the ciliate (*Orchitophyra stellarum*) was present in the gonads (Childs, 1970). Subsequently, I have found in the summers of 1976, 1978, and 1980, and even in January 1978, 1979, and 1980, that this failure of cells of infected individuals to react to glass has been readily demonstrable. In all four summers, it has appeared at high prevalence (about 30%) in animals from different sites near Woods Hole, Massachusetts (Table I). The very high correlation with ciliate infection (Table II) suggests that

TABLE II

Presence or absence of ciliates in seastars Asterias forbesi *and* A. vulgaris *combined.*

	Number examined	Number with ciliates	Percentage
Clumpers	57	0	0
Non-clumpers	24	22	92
Doubtful	6	1	17

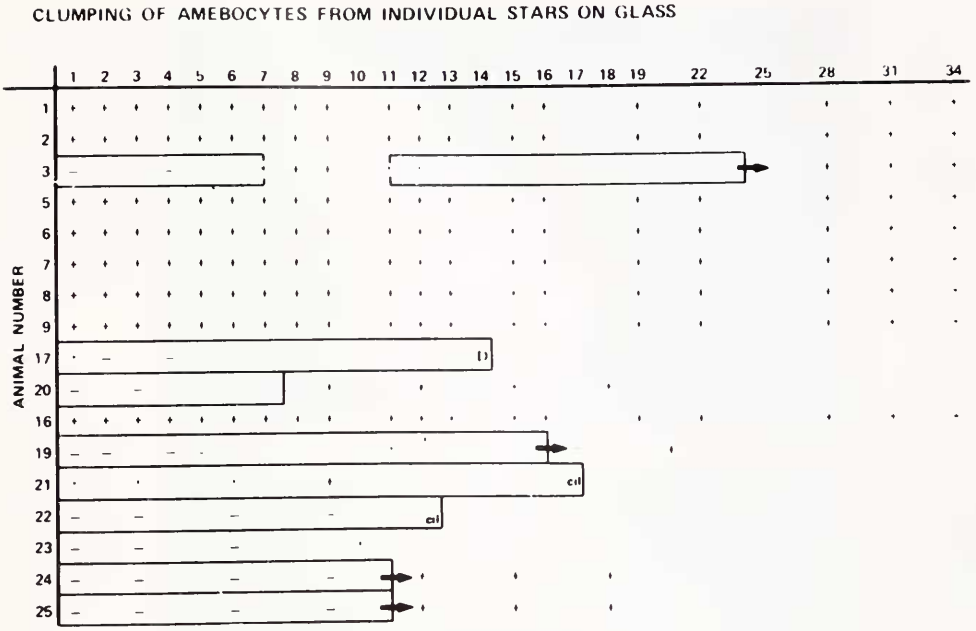

FIGURE 2. Clumping of amebocytes from individual stars on glass.

it is the direct cause of the abnormal behavior of these cells. Recovery from infection and return to normal glass-induced clumping frequently occurs within 10 days to 2 weeks (Taylor and Bang, 1978; Bang and Childs, unpublished) (Fig. 2).

The remarkable failure of the amebocytes to react to glass needs extensive study. While normal amebocytes begin to form clumps with each other within less than a minute after exposure to glass or plastic surfaces, cells from infected animals, whether obtained from the coelomic cavity or the water vascular system, remain unreactive (Fig. 3a,b,c). After centrifugation into a pellet, they can be readily resuspended by shaking and may be washed several times and even transferred into normal coelomic fluid without losing their abnormal surface properties. They do, however, gradually settle on the surface of a glass slide, and do ingest foreign material such as carmine or sea urchin cells injected into the coelomic fluid. However, the secondary reaction of sticking to each other is apparently inhibited completely. There seems to be a relation between heavy infections and the presence of the ciliates in the coelomic fluid. The similarity between amebocytes and mammalian platelets (Levin and Bang, 1964), noted at the turn of the century (Dekhuyzen, 1901; Loeb, 1902), is a still unexploited challenge for investigation of the surface properties of these cells.

III. Inflammation

A recent review by Ryan and Majno (1977) defined inflammation as a response of living tissue to local injury that leads to the local accumulation of blood cells and fluid. The overall process, seen against the broad perspective of evolution, is useful since its primary function is doubtless that of defense against microscopic invaders. Metchnikov (1892) in his "*Lectures on the Comparative Pathology of*

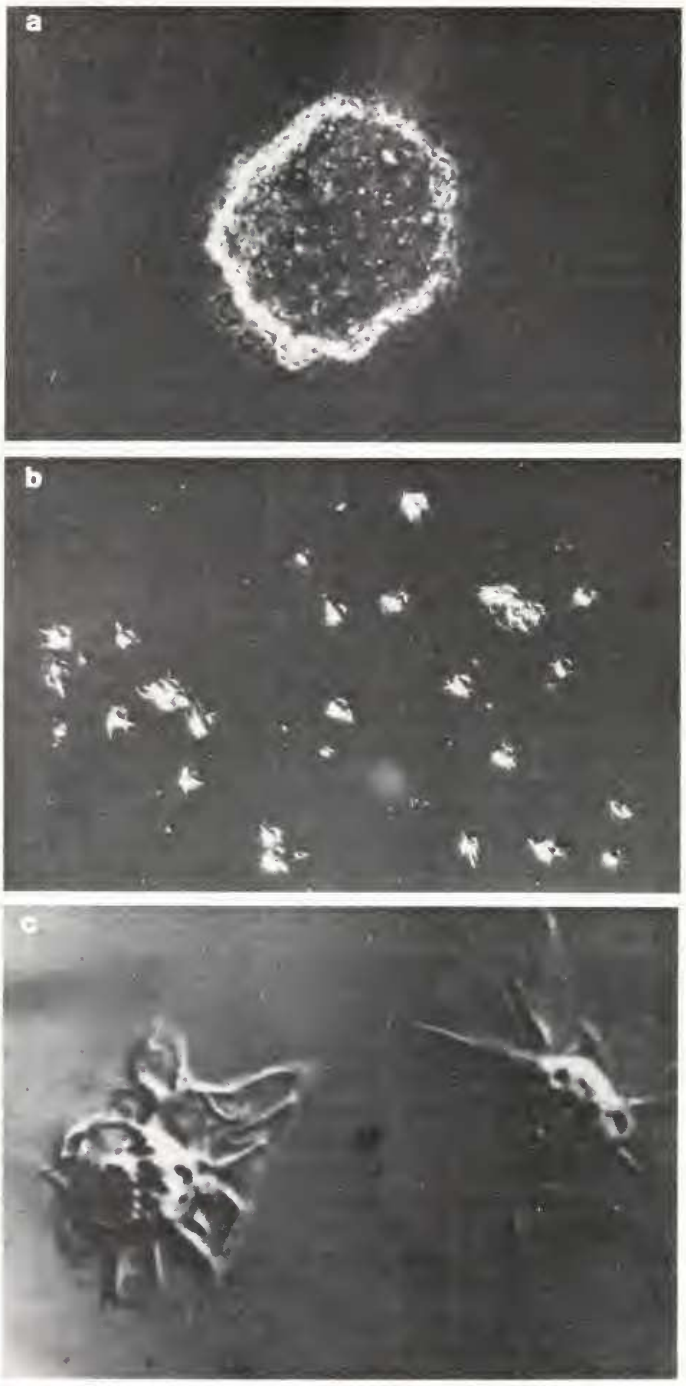

FIGURE 3. (a): Blood from a normal seastar forming a tight clump of amebocytes when exposed to glass. (b): Blood from a ciliate-infected seastar failing to clump on exposure to glass. (c): Individual amebocytes from ciliate-infected star showing normal petaloid form. (Nomarski optics) [Courtesy Dr. K. Edds.]

Inflammation" defined inflammation in terms of phagocytosis. At about this same time, when there was considerable interest in the excretion of foreign substances by invertebrates, Durham (1888) studied the emigration of ink-laden amebocytes from the seastar through the transparent papulae.

A. Response to sea urchin blood cells. We found that injection of sea urchin blood cells into the seastar was also followed by clumping of the urchin cells into cell plugs which appeared at the tips of the papulae 4–10 hours after injection (Reinisch and Bang, 1971). There was a concomitant marked reduction in the number of circulating host amebocytes, an effect that could be transferred to other

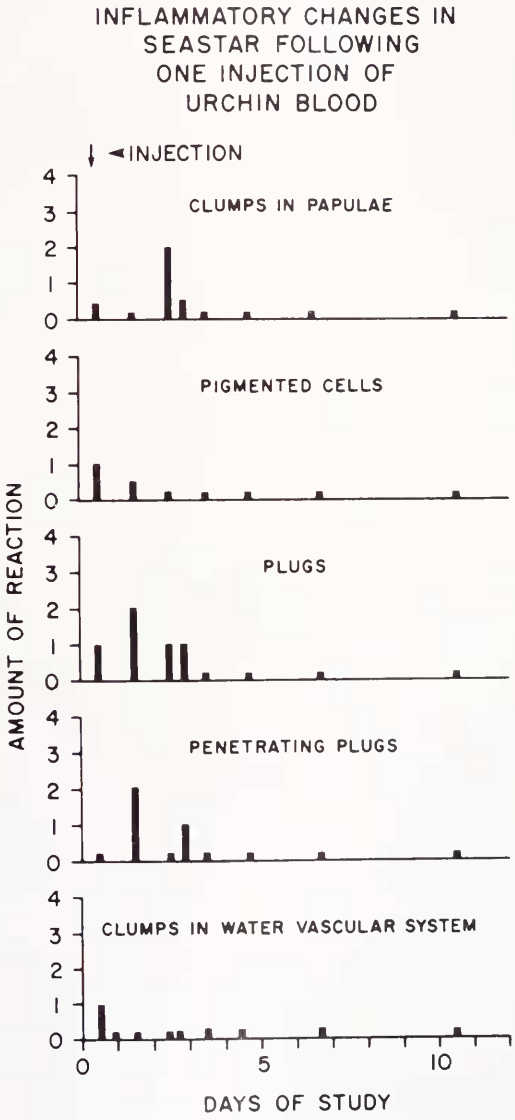

FIGURE 4. Inflammatory changes in seastar following one injection of urchin blood. Small bars represent negative findings.

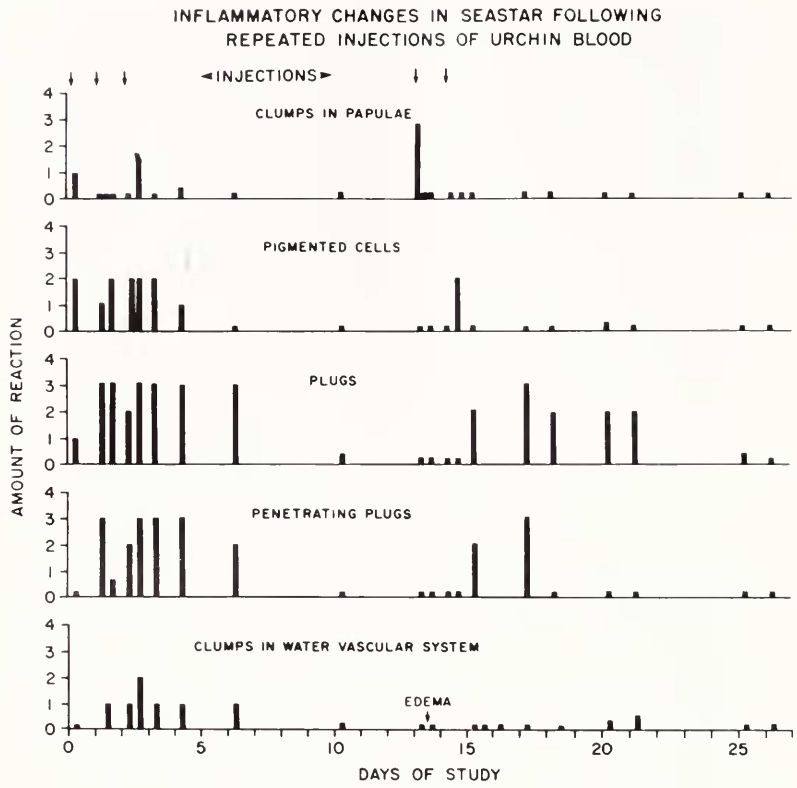

FIGURE 5. Inflammatory changes in seastar following repeated injections of urchin blood.

seastars by cell-free fluid from the injected star (Reinisch, 1974). This localization of the inflammatory response was followed by penetration of the foreign cells through the papulae, easily seen because of their bright red color. This color darkened as they were phagocytosed. They were carried through the papular tips in the same way that phagocytosed carmine particles are ejected through the tips. In order to quantify this process, five aspects of the reaction (Figs. 4 and 5) were followed in a number of small (6–10 g) seastars:

1) Clumping. The first clear sign of reaction was clumping of the urchin cells within the papulae. When 0.5 ml of freshly obtained urchin cells were injected into the arm of the seastar through a 27-gauge needle, the immediate response was minimal and usually transient; the plugs appeared later, in the papulae on both the aboral and the oral surface of the arm, usually in greater frequency on the latter.

2) Lysis. In some stars, there was a rapid (10–20 minutes) clearance of the red urchin cells (there are both red and white cells in the urchin blood, but the clearance of the colorless cells cannot be followed visually). During this rapid clearance, there was lysis of the pigmented cells. Lysis could be observed in fluid taken from injected seastars. The large red-pigmented cells rounded up, failed to send out their heavily pigmented pseudopods, individual granules became more apparent, then the granules were lysed so that the cell cytoplasm was pink; the cells finally disintegrated. In other stars, clearance required 1–2 days. We have not determined whether successive injections of urchin cells into the same seastar affect the lytic process.

3) Responses to single and multiple injections. Sequential study of individual animals showed that reaction to one injection of urchin cells was mild and usually cleared in 2–3 days (Fig. 4), that several injections were followed by greatly increased numbers of plugged papulae (Fig. 5), and that the plugged papulae would be resolved in two ways: (i) by passage through the wall of the papulae, leaving behind a hole if the mass of migrating cells was removed, and (ii) through gradual break-up within the papulae. The latter process occurred at a later time. The phenomenon was more easily followed over time in stars injected with carmine, in which circulating cells containing carmine were found as late as 4 weeks after injection.

Because of its poor adsorption to living tissue, Evans' blue dye (Noble and Gregerson, 1946) is used for determination of blood volume in mammals and for identifying localized areas of inflammation. Exposure of intact seastars for 20 minutes to a 1:1000 seawater dilution was followed by metachromatic purple staining of the feet and by blue localization wherever there had been external trauma to the skeletal spines (paxillae) and their covering epithelium. In 2 of 10 stars that were injected with urchin cells, both showed extensive diffuse staining of the aboral papulae of one limb (? the injected limb), but the other eight showed localized staining of papulae in which clumping and plugging were occurring. When 0.5 ml of this same concentration of the dye was injected into the coelomic cavity of normal stars, the dye remained within the cavity for a number of days, and several interesting events followed. During the first day, the dye gained entrance to the water vascular system but did not stain the feet purple. It was first picked up by circulating amebocytes in the papulae, then slowly by clumps of amebocytes in the feet. In addition, within one day the inner layer of the papulae stained blue and the inner and outer layer of the papulae seemed to separate, so that a cast of the inner layer became retracted from the outer wall of the papulae. By 3 days, the two epithelia rejoined and repaired.

4) Water vascular system. As stated earlier, the water vascular system is separate from the coelomic cavity. Yet some years ago we had seen, in the more transparent *Asterias rubens*, that injection of bacterial suspensions into the coelom was followed by typical tight clumping of the amebocytes within the antennae and/or feet (Bang, 1975). Clumps occurred at any place along the channel, but did not penetrate through the epithelium. Thus they could be considered a secondary result of the inflammatory process in the coelom. It is quite possible that the effect was mediated through the secretion of a neurohormonal product by the diffuse neural network. This clumping within the water vascular system was seen only occasionally following a single injection of urchin cells, but was frequently seen with the more extensive inflammation produced by several injections (Fig. 6).

5) Hemal system. The texts on echinoderm physiology refer to a third vascular system: a hemal system which seems to include the axial organ. According to Nichols (1969): "In the asteroids it is apparently not possible to distinguish a separate axial hemal system from the axial organ—the two seem to be one structure. This lies to one side of the stone canal. . . ." The similarity of the axial organ to the spleen was emphasized by Cuénot (1948), and more recently by Leclerc (1973, 1974). Millott (1966) has shown that destroyed cells are deposited in the axial organ of sea urchins. We have repeatedly dissected out this organ in the seastar following injection of foreign material, such as ciliate suspension or urchin cells, and have failed to detect any change in it. A recent paper (Kaneshiro and Karp, 1980) suggests that Tiedemann's bodies, which are outpouchings of the ring canal, may also participate in "immune reactions."

B. Other responses of seastars. 1) Edema. Edema is a well recognized com-

FIGURE 6. Clumps of amebocytes (see arrows) forming within extended feet of *Asterias*.

ponent of inflammation in vertebrates. It is much harder to define in invertebrates. However, an edema-like phenomenon (Bang and Lemma, 1962) did occur in this study, usually in the injected limb. From the calcareous spiny skeleton of the seastar, a series of separate spines (paxillae) project. These take a variety of forms, and along with the pedicellaria (small pincers) are covered by a thin transparent epithelium. This is usually fairly closely adherent to the skeleton and the spines, but when fluid accumulates between the skeleton and the epithelial layer, there is a ballooning of this epithelium over the paxillae so that individual spines are hidden. The response can also be easily recognized in the lateral spines on the oral surface of the individual arms. We have not been able to quantitate the response, but its gross presence was inescapable. It occurred in 5 of 14 animals in the first series, and more frequently in multiply injected animals. It occurred in the injected limb in 3 of 4 animals injected after a rest period of 11 days, and persisted for more than 48 hours.

2) Evocation of mucus-secretory substances. If this partially simplified model of inflammation is to be of any value in comparative studies, then it should mimic some of the other characteristics of vertebrate inflammmation. Inflammation of mucous membranes in vertebrates is usually accompanied by increased mucus secretion. During the last ten years, we have been interested in an invertebrate system, the urn cell complex of the coelomate *Sipunculus nudus*, which swims freely in the coelom and excretes mucus when bacterial infection occurs. These cell complexes can also be stimulated to secrete mucus *in vitro*, and we found that several mammalian sera and secretions contained mucus-stimulating substances (MSS) (Bang and Bang, 1972, 1979). In the summer of 1980, we found that seastar coelomic fluid normally contained no MSS, but that immediately following the injection of urchin cells into the seastar, a high titer of MSS appeared and persisted

as long as 24 hours (Fig. 7). This was accompanied by, or perhaps caused by, the destruction of the urchin cells. It will now be of great interest to determine whether the appearance of MSS is accompanied by abnormal secretion of mucus in the seastar itself.

3) Graft rejection is commonly used by vertebrate immunologists to study immunologic reactions to various histocompatibility antigens. Graft rejection is well known among invertebrates, and the work of Théodor (1966, 1970), who showed that the branches of one sea fan are rejected by those of another, is a classic instance of recognition of self and non-self. However, much of the work on invertebrates has been unduly influenced by the standard knowledge of vertebrate immunity. Among the echinoderms, Bruslé (1967) first showed that a skin graft could be transferred and maintained for months thereafter. Ghiradella (1965) showed that the implantation of the caecum of homologous species into *Asterias* and *Patiria* was followed by retention of the implant at least 1–5 weeks, but that heterologous species (actually different genera) eliminated the foreign tissue, either through the dermal brachiae (papulae) or possibly through the host stomach. Although host amebocytes occurred in numbers around damaged tissue, much of the tissue destruction seemed to occur in their absence. Hildemann and Dix (1972) and Karp and Hildemann (1976) have studied skin graft rejection in other seastars (*Protoreaster* and *Dermasterias*) and in a sea cucumber, *Cucumaria tricolor*. They also found that control integumentary autografts remained viable but that there was

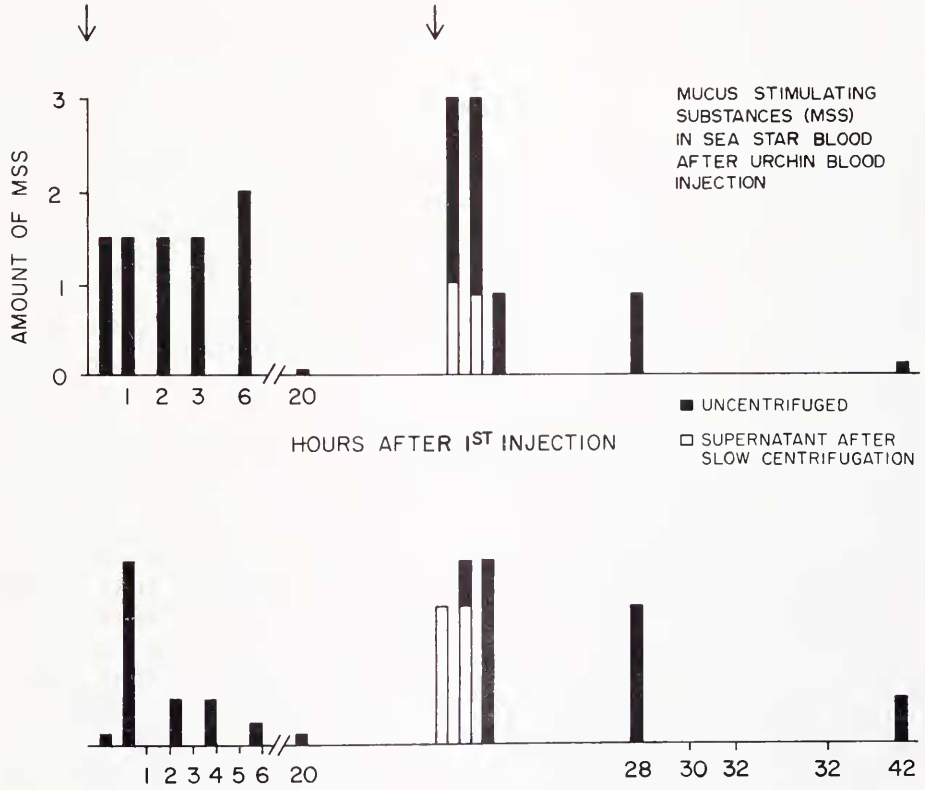

FIGURE 7. Mucus-stimulating substances (MSS) in seastar blood after urchin blood injection. Results shown represent same type experiment on two separate animals. Sea urchin blood by itself does not have mucus-stimulating activity.

slow, complete rejection of first-set heterologous grafts. To determine the specificity and memory in these graft rejections, they compared persistence in 17 first-set (213d), 5 second-set (44d), and 4 third-set (8d) grafts. Third party grafts were given to 5 animals, and 11 of these persisted longer than did the second party grafts. However, the series involved too few animals, and too little presently available histological detail, to evaluate the degree to which specificity and memory are linked, and thus to judge the possible similarity to the vertebrate system. Coffaro and Hinegardner (1977) have shown a genetic component to be important in grafting among sea urchins.

It is interesting in this regard to compare the capacity of ascidians to reject grafts by failing to fuse when grafted together. This system was extensively studied by Oka (1970) and Tanaka and Watanabe (1973). Fuke (1980) later found a counterpart reaction in which amebocytes killed each other on contact.

IV. Responses to ciliate infection

A. Background. In an early study (Bang, 1966), we found that a strong lysin against the marine ciliate *Anophrys* appeared in the serum of the marine worm *Sipunculus nudus* either during a spontaneous pox disease of *S. nudus* or after injection of foreign material into the animal. The *S. nudus* lysin was routinely effective against *Anophrys in vitro*. The sources of *Anophrys* were naturally infected *Carcinus* or *Cancer* crabs native to Roscoff in Brittany.

When seastars were injected with the blood of *Anophrys*-infected crabs, the parasite was effectively cleared from the coelom by 6 hours, and after subsequent injections was both cleared and lysed by 0.5 hours (Bang, 1975).

B. New findings. In 1981, however, we found that the rapidity of clearance and lysis of *Anophrys* after initial infection was directly related to previous exposure to marine ciliates. The first seastars used in the summer of 1981 were taken from a large stone aquarium in which a number of echinoderms were dying and were releasing a variety of marine ciliates into the running seawater. Nearly all of the seastars from this tank killed injected *Anophrys* within an hour or less. Removal of living stars to fresh running seawater for some weeks did not change this response. Only when seastars were newly collected by careful removal from mussel beds by hand, did we again routinely find animals which responded slowly.

Attempts to achieve lysis *in vitro* when *Anophrys* were added to coelomic fluid of stars that were strongly lytic *in vivo* consistently failed. This raised the question whether *in vivo* lysis was due to factors of fixed-tissue origin. The tip of an arm of a "lytic" star was cut off, carefully washed free of hepatopancreas and circulating cells, and placed in an open, plastic, minicentrifuge tube in a seawater bath. Fluid from the inside of the arm was then sampled periodically with a non-wettable plastic micropipet and placed on a slide. When *Anophrys* were added to this cell-free fluid, the ciliates were continually lysed (Bang, in press). The phenomenon was then comparatively tested and confirmed in tips of arms from clean and from other "contaminated" stars.

To summarize the lysin story: Freshly collected, previously uninjected, seastars take 6 or more hours *in vivo* to clear populations of injected *Anophrys* from the coelom. Destruction and lysis of these same ciliates occur *in vivo* within half an hour in animals injected the previous day, and in stars which are maintained under contaminated conditions. "Contamination" includes exposure to other ciliates (Grolière *et al.*, 1980), some of which infect the internal lining epithelium. *In vitro* lysis occurs in recently extirpated tips of arms that have been thoroughly washed of circulating cells, and seems to occur much more rapidly in those from "immunized" animals. The nature of the lysin, which in turn includes some autolysins,

is unknown. The *Sipunculus* lysin is known to be a molecule of 250,000 daltons (Bang and Shin, 1981).

A lysin for a variety of cells is liberated from seastars by heating the stars to 75°C (Bang and Chaet, 1959), but this may be related to the presence of saponins in seastar tissue (Owellen *et al.*, 1973).

Conclusions

We now know that despite the rather limited information on natural disease processes of marine invertebrates, the sea is a rich source of disease agents, each with different potentials for diseases in different hosts. Kinne (1980) points out that the more a particular species is studied, the more agents are found. These diseases will probably increase because of the current mixing of different animals from all parts of the world, either purposefully to develop disease-resistant stock, or inadvertently by transport of local species for improving commercial yields, or for study.

Although there remain many exciting, unexplored possibilities for the study of inflammation, injury, disease and immune responses in adult seastars, we must remember Libbie Hyman's (1955) preface to her volume on the echinoderms: "I also here salute the echinoderms as a noble group especially designed to puzzle the zoologist."

Acknowledgment

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